THE EFFECT OF ANTIBIOTICS ON THE VARIABILITY OF SOME PLANT PATHOGENIC FUNGI

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The undersigned hereby certify that they have read and recommend to the School of Graduate Studies for acceptance, a thesis entitled "The effect of antibiotics on the variability of some plant pathogenic fungi", submitted by J.W.S. Alcorn in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

The effect of several antibiotics on the variability of strains of the phytopathogenic fungi, Helminthosporium sativum, Pammel, King and Bakke; Colletotrichum linicolum, Pethyb. et Laff.; and Polyspora lini, Lafferty; in artificial culture was studied. Six antifungal antibiotics gliotoxin, griseofulvin, acti-dione, candicidin, nystatin, tyrothricin and one antibacterial antibiotic streptomycin were used. Variation was expressed by the formation of sectors and by the production of variant single spore colonies.

Each of the antifungal antibiotics was found in one or more instances to increase the variability of the three fungi. The increase in variation differed with the antibiotic, the concentration of the antibiotic, and the strain of the fungus used. Of the antibiotics studied griseofulvin was the most effective agent in increasing variation. It was demonstrated that with increase in antibiotic concentration, an increase in variation resulted. Growth of the three fungi was inhibited on antifungal antibiotic media. This inhibitive effect increased with rise in concentration. Only at concentrations reducing growth was an increase in variability observed.

Streptomycin, the antibacterial agent, had no apparent effect on either growth or variability.

As to the possible mechanisms in the production of variability, only the process of anastomosis was studied. Attempts to stimulate it in C. linicolum by antibiotics and bacterial isolates were unsuccessful.

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GENERAL INTRODUCTION

Antibiotics have for a number of years been widely used in the treatment of diseases of humans and animals. They have been applied in food preservation and animal nutrition with success. Recently these agents have shown promise in the control of plant diseases (1, 6, 27, 30, 31, 42, 48). In view of such extensive use of these substances it is important that any undesirable quality possessed by them be known.

A number of different effects of antibiotics on fungi have been observed. Their inhibition of fungal growth is well-known. Changes in pigmentation and spore production have been noted by Gossbard (20). Authors such as Brian, Curtis, and Hemming (8), Lachance (26), and Ward (4) have reported morphological modifications of various kinds. Little information as yet is available on the effect of these compounds on the variability of pathogenic microorganisms. An increase in genetic variation could well be a non-beneficial effect, if through it new more virulent races or biotypes were to arise. Is it then possible that antibiotics may increase variability?

Previous work has demonstrated that increased variability may occur in fungi through contact with toxic substances such as malachite green, mercuric chloride, ethyl mercury phosphate, (15), and 2,4-D (47). It is possible that antibiotics may act in a similar manner.

The purpose of the present investigation was to determine whether under the action of antibiotics the variability of plant pathogenic fungi in culture was increased.

The Plan and Scope of the Studies

The investigations have been divided into two parts. In part one, the studies were confined to three imperfect fungi, which are known plant pathogens. These were Helminthosporium sativum, Pammell, King, and Bakke; Colletotrichum linicolum, Pethyb et Laff; and Polyspora lini, Lafferty. The object was to study the effect of the antifungal antibiotics gliotoxin, griseofulvin, acti-dione, candicidin, nystatin, tyrothricin, and one antibacterial antibiotic streptomycin on the variability of the above fungi in artificial culture as measured by

1) The number of sectors or saltants produced
2) The number of variant colonies arising from single spores.

The second section has been devoted to the investigation of one of the possible mechanisms giving rise to variation, namely, anastomosis. Antibiotics, and bacterial isolates under varying conditions, were tested for their ability to stimulate this process in Colletotrichum linicolum.

PART I

INTRODUCTION

The three imperfect fungi chosen for the present studies have been observed by others to produce numerous and widely differing variants, usually occurring as sectors or patches, in colonies on artificial media (13, 24, 33). These variants as indicated by their stability were apparently genotypic rather than merely phenotypic. Variation frequency differed in different strains of the same fungus. Many investigators have shown that the frequency of variation in fungi can be altered by such factors as light, temperature, nutrient supply and the presence of certain salts or toxic substances (3, 14, 17, 38). An increase in the number of variants produced in an H. sativum culture, grown from a single spore on a toxic medium, has also been demonstrated (47). From this information it was decided to adopt the methods of measuring variability already mentioned under the plan and scope of the studies.

MATERIALS

1. Organisms studied

A preliminary survey was made of a large number of strains of the three imperfect fungi studied. The following were chosen for specific reasons, which are given.

Helminthosporium sativum

Strain 1. This strain was originally isolated from wheat and maintained at this laboratory as a stock culture for a number of years. It characteristically formed a dense dark black growth on potato dextrose agar*, sporulated readily, and when transferred regularly and kept on potato dextrose agar showed little tendency to sector or produce patches.

Strain 2. This isolate was obtained from barley seed by the author. It was similar to strain 1 in sporulation, but differed in being olive green to black in colour, and also in stability giving rise to sectors frequently. The growth rate, as measured by the average diameter of a colony over a given period of time, also was less than that of strain 1.

Through the use of the sparsely sectoring strain 1 it seemed that induced sectoring by antibiotics might be demonstrated, whereas by using strain 2 it was thought that an increase or decrease in the number of sectors produced might be detected.

Colletotrichum linicolum

The two strains of this fungus chosen for study were a conidial and a mycelial form.

Strain 1. This strain in culture was orange to pink in colour with little production of mycelium, and abundant production of spores. As a result it was designated a conidial type. In mass

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* Designated by the abbreviation P.D.A. in the remainder of the paper.

transfers this strain had shown a tendency occasionally to produce sectors dark in colour and of a mycelial nature.

Strain 2. This strain was obtained through the courtesy of Prof. A. E. Muskett, Plant Disease Division, Queen's University, Belfast. It was a typical mycelial form, being opposite in type to strain 1, by producing a dense dark-greyish mycelial growth and few conidia. A capacity to produce sectors was also observed in this strain on mass transfer.

In both strains, when single spore cultures were obtained, no sectoring was observed.

These two strains were included in studies to determine whether the conidial or the mycelial type was more variable under the influence of antibiotics. The tendency for the production of variants is normally from the conidial to the mycelial form. Is it possible that antibiotics can reverse this trend? The available strains of this fungus seemed to be especially suitable for work on this problem and hence were so used.

Polyspora lini

The two strains described here and incorporated in the studies were again a conidial and a mycelial type. Strain 1 was isolated by E. W. B. Ward of this laboratory from a sample of seed flax obtained from the Peace River district of Alberta. From this isolate, strain 2 was obtained as a variant.

Strain 1. The characteristic colony was flat, soft, profusely sporulating, with a comparatively poorly developed mycelium. At first it was white in appearance but soon turned black. It has a greasy yeast-like type of growth.

Strain 2. It appeared infrequently as a variant from strain 1 (24). In contrast, it may be described as firm, tough or leathery, often more or less raised and furrowed, and producing few spores. The colour varied from white to dark grey.

As with C. linicolum it was hoped to obtain evidence on the relative variability of the two strains of P. lini, and on the trend of variation of this fungus, under the influence of antibiotics.

The history of strain 2 is of interest since on plating out on P.D.A., when first obtained, it sectored regularly to give strain 1, the black type. This seemed unusual being the reverse of the trend mentioned previously in discussing C. linicolum. Henry (24) found in the strains studied no evidence of the grey hard form sectoring to give the black soft form. On obtaining many single-spore cultures of each strain the present author observed no sectoring of this nature to take place. Therefore, it was concluded that the initial isolate was a mixed culture.

2. Antibiotic materials

From table I it is observed that the antibiotics are produced by various groups of microorganisms. The source of an antibiotic gives no indication of its chemical nature,

Table I. A list of the antibiotics used in the investigation

	Antifungal Antibiotics	Produced by	Chemical Nature	Report of Anti-microbial Properties	Where Obtained
		FUNGI			
1.	Gliotoxin	Gliocladium fimbriatum Trichoderma viride Aspergillus fumigatus	$C_{13}H_{14}H_2S_2O_4$ (4)	Brian & Hemming (8) Weindling (42)	National Research Council, Prairie Regional Laboratory, Saskatoon.
2.	Griseofulvin	Penicillium griseofulvin P. janczewski P. patulum	$C_{17}H_{17}O_6Cl$ (4)	Brian et al.(7) Baron (4)	Brian, Imperial Chemical Industries, Butterwich Res. Laboratories, England.
		ACTINOMYCETES			
3.	Acti-dione	Streptomyces griseus	Diketone $C_{15}H_{33}NO_4$ (4)	Whiffen (44)	Upjohn Co., Kalamazoo, Mich.
4.	Candicidin	" "		Lechevalier, et al. (28)	N.R.C., Prairie Reg. Lab., Saskatoon.
5.	Nystatin (Fungicidin)	Streptomyces sp.	Amphoteric substance $C_{46}H_{83}NO_{18}$ (19)	Hazen & Brown (23)	" " " "
		BACTERIA			
6.	Tyrothricin	Bacillus brevis	Polypeptide	Stokes, et al. (36)	N.R.C., Prairie Reg. Lab.
	Antibacterial Antibiotic				
		ACTINOMYCETES			
7.	Streptomycin	Streptomyces griseus	Hydroxylated base (4) $C_{21}H_{37-39}N_7O_{12}$	Baron (4)	Upjohn Co., Kalamazoo, Mich.

antimicrobial spectrum, or potency. An attempt was made in the selection of the antibiotics to include as far as possible a representative sample of substances from the three classes of microorganisms, fungi, actinomycetes, and bacteria. This was done to obtain a general picture of the effect of antibiotics on the variability of fungi. Streptomycin, essentially an antibacterial agent, was chosen because it is in wide use in practical control, and to compare the effect of an antibiotic that is inactive against the fungi studied.

All antibiotic materials were stored in the cold room at approximately 5° C. to prevent as much as possible a loss of activity.

3. Culture materials

The fungi were grown throughout the entire studies on potato dextrose agar (P.D.A.).

GENERAL METHODS

Many antifungal antibiotics are essentially water insoluble. Hence organic solvents are necessary for preparing solutions. The following solvents were used: 95% ethanol, 75% acetone, and 50% N - propanol.

The particular antibiotic under study was dissolved in the chosen solvent and from this, solutions were prepared ten times more concentrated than required in the final media, by

by dilution with sterile distilled water. From each solution so prepared 1 ml. was transferred by sterile pipette to a petri dish, followed by 9 ml. of molten sterilized media. Thorough shaking and rotation were given to this mixture to ensure proper mixing, thus achieving dilution of each solution ten times, to give the required concentration. Controls were prepared by adding 1 ml. of the solvent at the highest concentration used in the experiment, instead of an antibiotic solution. Care was taken to ensure that the concentration of the solvent in the final medium did not exceed in any one case 2.5%. In preliminary experiments it was found that concentrations of the solvents above this amount affected the growth of the fungi.

As the frequency of variability in fungi has been observed by many authors to be altered by environmental factors, an attempt was made to place the different antibiotic treatments on a standardized basis.

1) The pH of the medium was brought in all cases to a level of pH 5.5. This was chosen since after autoclaving, the prepared P.D.A. medium had a pH reaction near 5.5. Also, fungi grow satisfactorily at this pH and such an acid reaction prevents appreciable contamination by bacteria. Finally, some of the antibiotics in the investigations were more stable under acid than under alkaline conditions.

A Beckman pH meter was used for all measurements of pH, the media being at a temperature of approximately 45° C. To adjust the hydrogen-ion concentration of a batch of medium, 10 ml. was taken from it and the pH of this was adjusted to 5.5 by the addition of N/10 HCl or N/10 NaOH. From this the volume of N/10 acid or alkali necessary to adjust the pH of the total volume to a pH of 5.5 was calculated. The effect of the addition of the antibiotic on the pH was disregarded.

2) All cultures under test for antibiotic effects were placed in an incubation cabinet with no source of illumination and maintained at a temperature of 250° C. Minor fluctuations occurred of only one or two degrees above or below this figure during the tests.

3) For certain fungi other investigators (10, 13, 29) have shown that the type of medium influenced greatly the frequency of variation. Therefore, the medium used was potato dextrose agar (P.D.A.), because it has been shown to have less effect than other media on the induction of sectors (13).

4) At first, inoculation* was made by means of a uniform mycelial mat cut from a single colony with a sterile cork borer, as used by Christensen in his many experiments on variation. Whether inoculation was carried out with mycelia or spores made little difference in sector production according to Brown (10). In tests made with the three fungi under study the same situation appeared to prevail. However, the method of inoculation, using a

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* Used here to mean the transfer of the fungus to another substratum.

mycelial mat, was abandoned for the following reasons. Firstly, it is probable that in this large amount of inoculum variability might already exist, and in the production of sectors the antibiotic might be acting as a selectant. Secondly, by this method of inoculation staling products might also be transferred and interfere with the antibiotic action. It is possible that the difference between sectoring and non-sectoring strains is the amount, or type, of staling products produced. Thirdly, the antibiotic has to act on a large amount of material when this method of inoculation is employed. Therefore, the method of inoculation adopted was by means of a spore suspension in sterile distilled water. This contained spores in such a concentration that a drop from a 3 mm. loop held about five to ten spores. Plates were inoculated at the centre, by touching lightly, with a 3 mm. loop charged in this spore suspension. All monosporous cultures, from which spore suspensions were prepared, were approximately fourteen days old.

5) Single-spore cultures were established for each strain. Subsequently, they were "single-spored" for three further generations to ensure stability of type, and to eliminate as far as possible excess variation in the cultures. It was observed even after this period of monosporous culturing that in some cases there was a noticeable difference between single spore cultures from the same parental source. Only those having characters similar to original type chosen were maintained, the others being discarded.

The technique adopted to establish a monosporous line was by pipetting approximately 1 ml. of an extremely dilute spore suspension into a petri dish, after which 10 ml. of molten water agar were added. The plate was rotated many times to ensure wide dispersal of the spores, and the layer of agar was then shallow enough to allow selection of one spore on germination. This was then cut out by means of a sharp pointed needle and transferred to a P.D.A. slant. All strains were maintained at room temperature on P.D.A., and "single-spored" at regular intervals throughout the investigation.

All experimental operations were carried out under aseptic conditions in a sterilized inoculation chamber.

EXPERIMENTS AND RESULTS

1. Antibiotic activity (Fungistatic)

In order to determine the concentrations at which the various antibiotics used inhibit the growth of the three fungi, preliminary tests by means of the agar-streak method of Waksman and Reilly (39) were made.

Table II

Antibiotic	Solvent
Gliotoxin	75% acetone (4)
Griseofulvin	75% acetone (2)
Acti-dione	Water (4)
Candicidin	50% N - propanol (37)
Nystatin	50% N - propanol (37)
Tyrothricin	95% ethanol (4)
Streptomycin	Water (4)

The above table lists the solvents used to dissolve the antibiotics. The antibiotic dilution range for this test was 250, 100, 50, 20, 10, 1, 0, parts per million. A maximum concentration of 250 ppm. was chosen since only a small amount of antibiotic material was available, and this automatically restricted the maximum concentration that could be used in the tests. Also, if no inhibition effect is visible at this concentration of 250 ppm. the antibiotic may be considered as being relatively inactive towards the organisms. Each check (0.0 ppm.) had the particular solvent, used for dissolving the antibiotic, incorporated in it.

Only one strain of each of the three fungi was tested, namely, H. sativum, strain 1; C. linicolum, strain 1; P. lini, strain 1. Duplicate petri plates of P.D.A. at pH 5.5, containing the antibiotic, were marked off into three segments. Spore

suspensions of the test organisms were applied, each within a marked sector, by streak inoculation using a needle bent at a right angle, producing a streak 1 cm. wide. Observations were made for growth after 5 days, and the results are given in tables III, IV, and V.

Table III. The inhibition action of the various antibiotics on H. sativum (1)

	Dilution of antibiotic (ppm.)						
Antibiotic	250	100	50	20	10	1	0
Griseofulvin	-	-	+	+	+	+	+
Gliotoxin	-	-	-	-	+	+	+
Acti-dione	-	-	-	+	+	+	+
Candicidin	-	-	-	-	-	+	+
Nystatin	-	-	-	-	-	+	+
Tyrothricin	±	+	+	+	+	+	+
Streptomycin	+	+	+	+	+	+	+

+ = Growth visible

± = Only a trace of growth

- = No growth

Table IV. The inhibition action of the various antibiotics on C. linicolum (1)

	Dilution of antibiotic (ppm.)						
Antibiotic	250	100	50	20	10	1	0
Griseofulvin	-	-	+	+	+	+	+
Gliotoxin	-	-	-	-	-	+	+
Acti-dione	-	-	-	-	-	+	+
Candicidin	-	-	-	-	-	+	+
Nystatin	-	-	-	-	-	+	+
Tyrothricin	±	+	+	+	+	+	+
Streptomycin	+	+	+	+	+	+	+

+ = Growth visible

± = Only a trace of growth

- = No growth

Table V. The inhibition action of the various antibiotics on P. lini (1)

	Dilution of antibiotic (ppm.)						
Antibiotic	250	100	50	20	10	1	0
Griseofulvin	-	-	+	+	+	+	+
Gliotoxin	-	-	-	-	-	+	+
Acti-dione	-	-	-	-	-	-	+
Candicidin	-	-	-	-	-	+	+
Nystatin	-	-	-	-	-	+	+
Tyrothricin	±	+	+	+	+	+	+
Streptomycin	+	+	+	+	+	+	+

+ = Growth visible

± = Only a trace of growth

- = No growth

H. sativum. In table III it can be seen that candicidin and nystatin exert a marked fungistatic effect on the germination of spores of this fungus at concentrations above 1 ppm. Gliotoxin is fungistatic between 10 and 20 ppm., while acti-dione and griseofulvin are fungistatic at higher concentrations. Although growth is observed at 250 ppm. dilution of tyrothricin only a trace of growth is observed at this concentration. Streptomycin shows no fungistatic activity at any of the dilutions used.

C. linicolum. From the data presented in table IV it is evident that four of the antibiotics, gliotoxin, acti-dione, candicidin, and nystatin inhibit growth at concentrations above 1 ppm. Griseofulvin is again fungistatic between 50 and 100 ppm. while tyrothricin and streptomycin show an antifungal activity that is similar to that recorded in table III for H. sativum.

P. lini. It is observed from table V that again the four antibiotics gliotoxin, acti-dione, candicidin, and nystatin suppress all growth at dilutions above 1 ppm. Acti-dione was shown by further tests not recorded to be extremely active allowing no growth of this organism even at 0.5 ppm., but growth took place at 0.1 ppm. Tyrothricin and streptomycin are found to act on P. lini, as they did on H. sativum and C. linicolum.

In general, it can be said from these tests that the three fungi showed an increasing sensitivity to the seven antibiotics in the order H. sativum, C. linicolum, and P. lini. In certain cases, mycelial and pigment production were stimulated.

2. The effect of antibiotics on variability of the three fungi as measured by the number of sectors produced

From the data given in tables III, IV, and V four concentrations for each antibiotic were chosen for the variability studies. The maximum dilution in this range where feasible was the greatest concentration allowing growth in the preliminary test on fungistatic activity. Where convenient, dilutions of 1/2, 1/10, and 1/100 of this amount made up the three other concentrations. An endeavour was made to incorporate in each range one antibiotic dilution which was similar throughout all experiments. This was done in order that a comparison might be made between the actions of antibiotics.

Centre point inoculation was made on P.D.A. containing antibiotic dilutions in petri dishes, by means of a spore suspension using a 3 mm. platinum wire loop. Readings were taken at 10, 15, and 20 days of the number of sectors produced per colony. Inhibition was measured by taking the average of two diameter readings of a colony made at right angles. The tests were replicated three times.

A. Helminthosporium sativum

H. sativum (1)

1. Gliotoxin and H. sativum (1)

This antibiotic was dissolved in 75% acetone. Dilutions of 10, 5.0, 1.0, 0.1, and 0 ppm. were made up in PDA, and inoculated with H. sativum as indicated in the general methods. The results are recorded in table VI.

It can be seen that at the high concentrations, 5.0 and 10.0 ppm., the number of sectors produced after 15 and 20 days' growth has been considerably increased compared with the check. With increase in concentration a relative increase in inhibition takes place giving decrease in colony size. Sectoring increases through 10, 15, to 20 days, this being most noticeable at high concentrations. The increase in the number of sectors occurs only at dilutions which inhibit growth appreciably.

The sectors produced were lighter in colour, with a very well developed mycelium which was observed in some cases to be 2 - 3 mm. taller than that of the main colony. They were formed only at the perimeter, measuring about 5mm. in width. On making transfers from some of these sectors they maintained their mycelial character.

On one plate at 5ppm. it was noted that two contaminant bacterial colonies showed a large amount of sectoring. Whether, or not, this was due to antibiotic action is not known.

Table VI. The effect of gliotoxin on the growth and sectoring of H. sativum (1)

Antibiotic Concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0.7	76.0	1.0	83.0	2.0	90.0
0.1	1.0	81.5	1.0	83.2	1.0	90.0
1.0	0	73.0	0	75.6	0	77.3
5.0	3.0	49.1	7.7	57.1	10.3	59.5
10.0	2.0	39.6	11.0	47.3	13.7	50.33

Table VII. The effect of griseofulvin on growth and sectoring of H. sativum (1)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	82.5	0	88.3	0	90.0
0.1	0	82.5	0	88.0	0	90.0
1.0	1.7	62.5	2.3	72.0	4.0	74.0
5.0	2.3	38.0	8.0	46.5	10.7	50.8
10.0	6.7	27.8	17.0	37.4	18.0	38.5

No attempt was made to identify the bacterium. The colonies were yellow, opaque and smooth, with the sectors being lighter in colour.

2. Griseofulvin and H. sativum (1)

The same procedure was followed as in the previous section. The same solvent and the identical concentration range were used to determine the effect of griseofulvin on H. sativum. The data are recorded in table VII.

After ten days a difference between the number of sectors induced by different concentrations of the antibiotic is evident. A similar trend as was found with gliotoxin in the last test is observed, i.e., the highest concentration induced the largest number of sectors. It is worthy of note that sectoring only occurs when inhibition takes place. Sectoring increases over 10, 15, and 20 days only at concentrations causing a decrease in growth. The effect of concentrations of 5 and 10 ppm. is pronounced after ten to twenty days.

The nature of the sectors was similar to that in the last test, except after 20 days it was noted that white variants appeared at 10 and 5 ppm., while no white sectors were observed at dilutions of 0, 0.1 and 1.0 ppm. The width of sectors, measured at the perimeter, was from 3 - 10 mm.

From a comparison of H. sativum (1) at 0 ppm. in tables VI and VII, it is evident that there is a difference in sectoring

capacity even between single spore cultures from a monosporous culture. Though the same solvent had been used in both tests, sectoring occurs at 0 ppm. in the gliotoxin test but not in the griseofulvin test even after 20 days.

A bacterial colony at 5 ppm. was noted have a few sectors similar to those already described.

3. Acti-dione and H. sativum (1)

The methods used here were identical to those used in the last two tests. The solvent was water and again the dilution range was 10, 5, 1.0, 0.1, and 0 ppm. Table VIII gives the results obtained.

In general, compared with the other two experiments, there is a similar decrease in growth with increase in concentration, but although an increase in the number of sectors takes place it is slight. The variants produced as in the other two tests were noticeable only on medic causing a considerable inhibition of growth. Only one of the variants induced was white in colour. This appeared in a plate containing 5 ppm. of the antibiotic.

4. Candicidin and H. sativum (1)

The dilution range 5.0, 1.0, 0.5, 0.1, 0.01, and 0 ppm. was prepared as in previous experiments using 50% N-propanol as a solvent. The results from this test are recorded in table II.

Table VIII. The effect of acti-dione on the growth and sectoring of H. sativum (1)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	81.0	0.3	86.2	0.3	88.5
0.1	0	80.5	0.3	86.0	0.3	87.9
1.0	0.7	68.0	2.7	75.1	3.3	78.6
5.0	1.3	50.1	3.0	58.1	3.3	62.0
10.0	0.3	41.2	2.7	46.7	3.0	49.3

Table IX. The effect of candicidin on the growth and sectoring of H. sativum (1)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	60.0	0	76.0	0	81.0
0.01	0	61.0	0	75.8	0.7	82.1
0.1	0	54.0	0.7	68.2	1.3	74.3
0.5	0.3	42.1	2.0	56.0	3.0	63.0
1.0	0	35.5	2.0	43.6	2.7	50.5
5.0	0.3	21.1	2.0	32.0	2.0	42.2

Little or no sectoring occurs at the 10 day mark. A small increase in sectoring with ascending antibiotic concentrations is observed. The trend is thus similar to that shown in previous tests. No variants are observed in the check at 15, 10, or 20 days. After 20 days a slight decrease in the number of sectors produced takes place with increase of antibiotic concentration from 0.5 through 1.0 to 5.0 ppm. Whether this is due to antibiotic action or experimental error is not known. Yet a decrease in growth results over this concentration range.

All variants were characterized by an abundant production of mycelium, some being white, but most of them were only lighter in colour than the main colony. A few of the variants were transferred to non-antibiotic media, where all except one maintained their original characteristics.

5. Nystatin and H. sativum (1)

The same procedures as already employed were used, but 50% N-propanol was employed as the solvent. A change in dilution range was made giving concentrations of 1.0, 0.5, 0.1, 0.01 and 0 ppm. for this particular experiment. The results are given in table X.

It is apparent that the pattern of variability observed in previous experiments is to some extent again being repeated in this test. After 20 days' growth a significant difference is evident in the number of sectors produced at different

antibiotic concentrations, although no appreciable difference could be detected after 10 and 15 days. With increase in antibiotic concentration a corresponding growth inhibition takes place.

The sectors were typically mycelial as described before. Two white variants were produced at both the 1.0 and 0.5 ppm. concentrations.

6. Tyrothricin and H. sativum (1)

The general methods of previous experiments were again followed, with 95% ethanol being used to dissolve the antibiotic. From table IV the concentration range of 250, 100, 10, 1, and 0.0 ppm. was employed. Table XI indicates the results noted. If tyrothricin is compared with the other antifungal antibiotics studied, on the basis of activity per ppm., it may be considered relatively inactive. In spite of this at the high concentrations employed there is a remarkable reduction in growth. This antibiotic in its action produced a similar effect to other antibiotics used, namely that with increase in concentration within the range employed it tends to induce the production of more variants. It is observed from the table in a clear-cut manner that sectoring is only induced when inhibition occurs. An increase in the number of sectors produced is achieved up till 15 days, but even although growth continues after this no further increment is obtained.

Variants were mostly mycelial in nature being lighter in colour than the main colony, but white types were observed at dilutions of 10, 100, and 250 ppm.

Table X. The effect of nystatin on the growth and sectoring of H. sativum (1)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	2.3	81.0	2.7	84.0	2.7	86.0
0.01	4.2	74.5	5.0	76.0	5.3	79.0
0.1	0.7	80.0	2.0	80.2	3.0	81.1
0.5	2.3	57.4	4.7	64.0	7.7	67.0
1.0	2.0	40.4	5.0	48.1	8.7	51.5

Table XI. The effect of tyrothricin on the growth and sectoring of H. sativum (1)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	72.5	0	76.0	0	78.0
1.0	0	78.8	0	80.0	0	80.6
10.0	4.3	42.0	8.0	50.7	8.0	51.3
100.0	2.3	18.6	9.0	25.3	9.0	28.7
250.0	2.3	16.7	9.3	22.3	9.3	25.8

7. Streptomycin and H. sativum (1)

This antibiotic, which has been widely used as a bactericidal agent, was made up in dilutions of 250, 100, 10, 1 and 0 ppm. in sterile distilled water.

Streptomycin is active against downy mildew fungi but inactive against the fungi studied. Although a slight reduction in growth was obtained at high concentrations only one sector (an average of 0.3 over 3 replicates) was observed in the test; this was at 250 ppm. This antibiotic therefore has no appreciable effect on sectoring at the concentrations employed.

Table XII. The effect of streptomycin on the growth and sectoring of H. sativum (1)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	80.1	0	86.0	0	87.2
1.0	0	81.2	0	87.2	0	88.0
10.0	0	81.6	0	86.5	0	87.4
100.0	0	77.2	0	81.0	0	84.0
250.0	0	76.0	0	79.1	0.3	82.2

H. sativum (2)

Experiments with H. sativum (2) were carried out in the same way as with H. sativum (1) using all seven antibiotics. Under test this strain proved to be too variable to yield the required information, namely whether an increase or decrease in sectoring would be induced by antibiotic action.

From table XIII, which records the results of the action of griseolfulvin on strain 2, there is an apparent decrease in the number of variants with increase in antibiotic concentration. All six antifungal antibiotics tested in fact produce the same trend. This would then appear to be an effect in complete contrast to that demonstrated with strain 1.

However, this may be explained on the basis of the hypothesis that for a particular colony size the perimeter length will limit the total number of sectors that can be detected. If a decrease in colony size takes place, for example because of an antibiotic inhibition effect, a corresponding reduction in this maximum number of sectors will result. It is suggested that this strain has in all probability reached its maximum capacity for sectoring. Therefore what is perceived in the table is not a decrease in variation but merely a limitation laid on the number of sectors produced by the colony size. Also, as the strain was too unstable to show an induced increase, no definite conclusions could be reached on the action of antibiotics on the variability of this strain.

Table XIII. The effect of griseofulvin on the growth and sectoring of *H. sativum* (2)

Antibiotic concentration ppm.	15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	18.0	40.0	18.0	40.8
0.1	22.6	44.6	22.7	46.9
1.0	13.6	36.25	16.7	38.5
5.0	12.6	22.80	12.6	25.4
10.0	11.3	20.0	13.3	24.3

Table XIV. The effect of streptomycin on the growth and sectoring of *H. sativum* (2) after 15 days

Antibiotic concentration ppm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	16.5	37.9
1.0	17.6	39.3
10.0	20.0	38.5
100.0	21.0	39.0
250.0	17.0	38.0

Table XIV gives the results of the action of streptomycin on H. sativum (2). Little or no effect on either growth or sectoring is observed under the action of this antibiotic.

B. Colletotrichum linicolum

The two strains of this fungus were studied using the same procedures adopted in experiments with H. sativum. Similar solvents were employed in preparing the different antibiotic concentration ranges.

The antibiotics gliotoxin, acti-dione, candicidin, nystatin, tyrothricin, and streptomycin at the concentrations indicated below were found to be ineffective in inducing sectoring in either strain 1 (the conidial type) or strain 2 (the mycelial type).

1. Gliotoxin 1, 0.5, 0.1, 0.01, and 0 ppm.
2. Acti-dione 5, 1, 0.5, 0.1, and 0 ppm.
3. Candicidin 5, 1, 0.5, 0.1, and 0 ppm.
4. Nystatin 5, 1, 0.5, 0.1, and 0 ppm.
5. Tyrothricin 250, 100, 10, 1, and 0 ppm.
6. Streptomycin 250, 100, 10, 1, and 0 ppm.

In these experiments sectors were found only rarely although a large number of cultures were examined. The antibiotics at high concentrations were effective in decreasing growth of C. linicolum. An example of this is provided by the action of tyrothricin, the results of which are recorded in table XV. At high concentrations of the antibiotics there was in some instances an increase in the

Table XV. The effect of tyrothricin on the growth of C. linicolum as measured by the average diameter of a colony in mm.

Antibiotic concentration ppm.	Strain 1		Strain 2	
	10 day	20 day	10 day	20 day
0	90.0	90.0	90.0	90.0
1.0	90.0	90.0	90.0	90.0
10.0	76.6	90.0	73.7	90.0
100.0	25.3	52.8	29.0	54.8
250.0	14.3	36.7	22.0	41.7

intensity of the orange pigmentation in strain 1, and a stimulation in mycelial production in strain 2. This was especially noticeable at concentrations of 250 and 100 ppm. of tyrothricin.

Griseofulvin proved an exception in its effect on the variability of C. linicolum (tables XVI and XVII). Under the action of the higher concentrations of this antibiotic a few mycelial sectors were produced by strain 1. In the case of strain 2 a definite trend of increase in variability with increase in concentration of griseofulvin may be noted from the data in table XVII. The strain 2 saltants were less mycelial in character than the parent culture, some even being orange in colour a characteristic commonly associated with conidial forms of C. linicolum. This would then appear to be a reverse in the normal pattern of saltation. Similar results were obtained in a second test. In general it may be concluded that strain 2 was more variable than strain 1 under the influence of griseofulvin.

C. Polyspora lini

The methods employed in the work with P. lini were identical to those used in previous studies. Hence only the results will be considered in the following section.

1. Gliotoxin and P. lini (1) and (2)

The effects of gliotoxin on the variability of two strains of P. lini are given in tables XVIII and XIX. Practically no sectoring is observed in the two strains after 10 days. However,

Table XVI. The effect of griseofulvin on the growth and sectoring of *C. linicolum* (1)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	90.0	0	90.0	0	90.0
0.1	0	90.0	0	90.0	0	90.0
1.0	0	61.2	0	85.0	0	90.0
5.0	0	35.0	0	65.4	0.7	84.7
10.0	0	24.6	0	45.0	0	68.7
20.0	0	17.7	0	34.2	0.3	39.7

Table XVII. The effect of griseofulvin on the growth and sectoring of *C. linicolum* (2)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	90.0	0	90.0	0	90.0
0.1	0	90.0	0	90.0	0	90.0
1.0	0	62.6	0	87.3	0.3	90.0
5.0	0	31.2	0.7	60.7	1.0	85.0
10.0	0	21.0	1.0	39.5	2.3	67.5
20.0	0	18.1	1.3	35.1	2.3	40.0

Table XVIII. The effect of gliotoxin on the growth and sectoring of P. lini (1)

Antibiotic concentrations ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	35.8	2.0	55.0	2.7	68.0
0.01	0	35.7	1.7	55.0	1.3	68.0
0.1	0	35.7	2.3	54.4	3.7	68.0
0.5	0.7	34.8	1.7	46.0	3.3	56.1
1.0	0.3	30.5	3.0	46.0	4.3	56.8

Table XIX. The effect of gliotoxin on the growth and sectoring of P. lini (2)

Antibiotic concentrations ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	25.2	0	40.0	0.3	51.0
0.01	0	24.8	1.0	39.5	1.0	51.0
0.1	0	24.3	2.0	39.0	2.0	51.0
0.5	0	20.5	4.3	35.8	4.3	43.0
1.0	0	19.7	4.7	34.5	5.0	42.1

after 15 and 20 days, there is a considerable overall difference between strain 1 and 2 in the number of sectors produced. A small increase occurs in strain 1, whereas a decided increment is produced in strain 2, between 0 and 1.0 ppm. From statistical analysis the difference between concentrations was found to be insignificant at the 5.0% level in strain 1 (table XVIII), and significant at the 1.0% level in strain 2 (table XIX). Although the total number of sectors produced at the highest concentration was practically similar in both strains, only in strain 2 was an increase in variability achieved.

Sectors produced in strain 1 were distinct but not of the grey tough form. They differed from the main colony in the amount and distribution of black pigment. Often these sectors were observed to extend beyond the perimeter of the main colony giving it an asymmetrical shape. From this it appeared that the variants in certain cases were possibly less sensitive to the action of the antibiotic than their parent. In strain 2 the sectors were lighter in colour but less compact than the original culture.

2. Griseofulvin and P. lini (1) and (2)

The results for this experiment are given in tables XX and XXI. The most noticeable feature is the large number of sectors produced at 20 ppm. after 15 and 20 days in strain 1. In contrast, few sectors are produced by strain 2 at the corresponding concentration and time. A strain difference directly opposite to that shown in the preceding test with gliotoxin is evident. This

Table XX. The effect of griseofulvin on the growth and sectoring of P. lini (1)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	35.5	2.0	48.8	4.0	60.0
0.1	2.0	31.3	2.0	45.8	3.3	56.2
1.0	0	31.3	1.7	45.8	4.0	56.0
5.0	0	26.0	4.0	40.8	5.7	51.0
10.0	0	21.4	7.7	34.0	9.0	41.8
20.0	2.7	18.3	9.7	27.8	13.0	38.0

Table XXI. The effect of griseofulvin on the growth and sectoring of P. lini (2)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	25.0	1.7	39.8	2.3	51.8
0.1	0	25.7	2.0	39.5	3.0	50.5
1.0	0	23.3	1.7	37.5	1.7	48.6
5.0	0	17.0	1.7	26.7	3.0	33.5
10.0	0	14.5	0	21.7	1.7	29.7
20.0	0	13.5	0	20.3	1.7	24.5

was corroborated by statistical analysis of the results obtained for both strains at the 20 day mark, the differences for strain 1 being significant at the 1.0% level, and those for strain 2 at the 5.0% level. The 13 sectors per colony recorded for the black soft type, under the action of 20 ppm. griseofulvin, after 20 days, is the largest number observed to occur in any test with P. lini. In these tests there was a considerable reduction in growth at the high antibiotic concentrations used.

In strain 1 the variants were similar to those already described in the preceding test. It was of interest that in one of the replicates of strain 1 at 20 ppm. a sector of strain 2 type was detected. This, however, may not have resulted from antibiotic action. The saltants in strain 2 were mostly lighter in colour than their parent, a few being darker in colour. All variants of strain 2 retained the tough quality of the parental strain.

3. Acti-dione and P. lini (1) and (2)

In both strains no sectors occur at 0.1 ppm. although sectoring is evident at other antibiotic dilutions (tables XXII and XXIII). This may be explained by the colony size being so decreased by antibiotic action that no sectors could be detected. Excluding the figure for the 0.1 ppm. treatment, the results at the 20 day level for strain 1 and strain 2 were found not to be significant at the 5.0% level. Therefore acti-dione apparently did not increase variability over that of the check in either strain 1 or strain 2.

Table XXII. The effect of acti-dione on the growth and sectoring of P. lini (1)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	31.0	1.7	45.0	3.0	58.7
0.001	0	32.0	3.0	48.0	3.0	58.0
0.01	0.3*	22.0	2.7*	33.25	3.3*	56.1
0.05	0.7	14.0	2.0	21.0	3.0	28.0
0.01	0	8.6	0	10.7	0	13.0

* 1 white tough sector

Table XXIII. The effect of acti-dione on the growth and sectoring of P. lini (2)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	22.75	1.7	39.5	1.7	48.0
0.001	0	21.75	1.7	31.5	1.0	43.0
0.01	0	17.00	2.0	25.0	2.7	29.5
0.05	0	10.1	1.0	18.5	2.7	19.3
0.1	0	8.5	0	10.0	0	10.3

At 0.01 ppm. the black soft form under test gave rise to a sector of the grey hard form. The two instances of this type of variation noted were the only occasions on which this was observed by the author, though many different strains and isolates of P. lini had been examined.

4. Candicidin and P. lini (1) and (2)

Tables XXIV and XXV record the results obtained for the action of this antibiotic on the two strains of P. lini. A decrease in growth of both strains occurs at high concentrations of candicidin. After 15 and 20 days growth sectoring is irregular in strain 1 with no definite trend apparent. Results at the 20 day level, however, were subjected to statistical analysis and found to show a significant difference at the 5.0% level. Because of irregularities in sectoring it was determined by the method of "least significant difference" that only between the check and the highest concentration employed did a significant difference exist. Thus compared with the check an increase in the number of sectors occurred at 1.0 ppm., the highest concentration used. In strain 2 a decided increase occurs in the number of sectors produced at high concentrations compared with 0.0 ppm. A statistical analysis of the recorded readings at the 20 day mark showed a significant difference between treatments at the 1.0% level. The variability of both strains was affected by this antibiotic, but strain 2 was influenced to a greater extent than strain 1.

Table XXIV. The effect of candicidin on the growth and sectoring of *P. lini* (1)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	30.8	2.7	49.0	2.7	59.0
0.01	0	29.5	2.0	44.6	3.7	57.3
0.1	0	29.2	0.7	44.0	1.0	58.5
0.5	0	24.7	3.0	40.25	3.0	52.0
1.0	0	18.5	1.0	33.0	4.0	43.3

Table XXV. The effect of candicidin on the growth and sectoring of *P. lini* (2)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	27.3	0	43.6	0	51.7
0.01	0	26.3	0.7	42.4	0.7	52.0
0.1	0	24.7	1.7	39.0	1.7	48.5
0.5	0	20.0	2.0	35.5	3.3	44.0
1.0	0	16.0	3.3	28.6	3.3	38.0

The sectors resembled those produced under the action of other antibiotics already studied.

5. Nystatin and P. lini (1) and (2)

From tables XXVI and XXVII it is observed that little reduction in growth occurs in either strain 1 or strain 2 of P. lini except at 5.0 ppm. of this antibiotic. In both strains sectoring is observed to take place only after 10 days growth. The small differences between treatments, in the number of sectors in strain 1 produced at the 20 day level, was found to be significant at the 1.0% level. In strain 2 all antibiotic treatments show an increase in the number of sectors compared with the check. An apparent trend of increase in variation with increase in concentration occurs over the dilution range 0, 0.01, 0.1, and 0.5 ppm. but decrease in this tendency takes place at 1.0 and 5.0 ppm. However, the data show an insignificant difference between treatments at the 5.0% level. Nystatin at the dilutions employed increased the production of sectors over the check in strain 1 but not in strain 2. The strain difference observed is similar to that shown under the influence of griseofulvin.

The saltants were similar to those already described in earlier experiments.

Table XXVI. The effect of nystatin on the growth and sectoring of *P. lini* (1)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	36.0	1.7	55.0	1.7	62.0
0.01	0	35.5	2.3	54.0	2.7	62.8
0.1	0	35.3	1.0	55.0	1.0	62.0
0.5	0	33.9	2.0	51.0	3.0	61.8
1.0	0	33.8	2.0	50.0	2.7	61.0
5.0	0	28.0	4.0	47.0	4.0	54.1

Table XXVII. The effect of nystatin on the growth and sectoring of *P. lini* (2)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	26.8	0	40.7	0.7	52.3
0.01	0	26.1	2.0	40.7	2.3	52.0
0.1	0	25.3	3.0	40.5	3.3	52.0
0.5	0	26.0	2.7	40.6	3.0	51.6
1.0	0	22.2	2.0	39.0	2.0	51.0
5.0	0	12.2	1.0	17.4	1.3	26.0

6. Tyrothricin and P. lini (1) and (2)

The inhibition of growth of both strains at 250 ppm. is considerable (tables XXVIII and XXIX). At this concentration sectors were not detectable because of the small colony size. Only one sector or an average of 0.3 for three plates is recorded in the experiment with strain 2, and that is in the check. Strain 1 shows an appreciable amount of sectoring with an apparent increase at 10 and 100 ppm., after 10 and 20 days growth. If the result for 250 ppm. is excluded the treatments show an insignificant difference at the 5.0% level. From these findings it is concluded that tyrothricin has no effect on variability as measured by the number of sectors produced.

All variants detected were similar in character to those already described in previous experiments.

7. Streptomycin and P. lini (1) and (2)

The results are given in tables XXX and XXXI. No appreciable growth reduction takes place at any of the dilutions used. Sectoring occurs in both strain 1 and strain 2 but little difference can be observed between the different treatments in the number produced. Streptomycin has thus little apparent effect on either the growth or sectoring of the strains of P. lini studied.

Table XXVIII. The effect of tyrothricin on the growth and sectoring of P. lini (1)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	23.6	0	40.7	0.3	55.0
1.0	0	24.0	0.7	43.0	0.7	50.3
10.0	0	19.6	1.7	35.4	1.7	35.6
100.0	0.7	15.3	1.7	19.5	1.7	23.0
250.0	0	8.6	0	13.6	0	15.0

Table XXIX. The effect of tyrothricin on the growth and sectoring of P. lini (2)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	19.1	0	35.0	0.3	40.2
1.0	0	18.3	0	30.0	0	37.0
10.0	0	13.8	0	21.3	0	27.1
100.0	0	8.4	0	14.1	0	18.0
250.0	0	5.5	0	10.0	0	13.0

Table XXX. The effect of streptomycin on the growth and sectoring of P. lini (1)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	24.8	0	40.8	0.3	52.0
1.0	0	25.1	0.3	39.1	0.3	50.4
10.0	0	24.5	0	38.0	0.3	50.6
100.0	0	24.0	0	37.6	0	46.3
250.0	0	24.2	0.3	38.0	0.7	47.5

Table XXXI. The effect of streptomycin on the growth and sectoring of P. lini (2)

Antibiotic concentration ppm.	10 days		15 days		20 days	
	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.	Av. no. of sectors per colony	Av. diam. of colony mm.
0	0	19.0	0.3	32.0	0.3	38.3
1.0	0	19.0	0	31.7	0	37.8
10.0	0	19.2	0	31.6	0	37.3
100.0	0	19.0	0	30.9	0.3	36.3
250.0	0	18.1	0.3	30.2	0.3	35.3

3. The effect of antibiotics on variability as measured by the number of variant colonies arising from single spores grown on media containing the antibiotics

This experiment was set up as an additional method of measuring variability, and to check the findings of the "sector" test. It consisted essentially of growing cultures from single spores on media containing antibiotic dilutions. These cultures were then examined for the production of variants by using an atomizer spray technique.

In the "sector" tests there was a possibility that the inoculum consisting of a number of spores might have contained variants. From experiments in this section it may be determined whether the antibiotic was acting as a selectant on these variants.

Employing solvents used in previous studies, two dilutions and a check were made up for each antibiotic. These were incorporated in potato dextrose agar slants, containing 5 ml. of media. Single spores from monosporous cultures were transferred to these slants which were then incubated at a constant temperature of 25° C. for approximately 14 days. A one ml. dilute spore suspension in sterile distilled water was prepared from each slant. This was diluted 25 times with sterile distilled water before adding 2 ml. by pipette to a sterile atomizer flask. The flask was then filled with distilled water giving further dilution. The spores in suspension were then sprayed on to 5 petri dishes, each containing 10 ml. of P.D.A. media. After incubation for 5 days at

25° C. the plates were examined for variant colonies. Complete aseptic technique was used throughout all experiments.

If the spore suspension was extremely dilute and care taken with spraying it is possible that the colonies obtained would be formed from single spores. Confirmation of this hypothesis was obtained by an examination of a large number of plates seeded with H. sativum.

A. Helminthosporium sativum

Only strain 1 was studied in these experiments. Strain 2 was not included because of its extreme unstability. It was noted in preliminary tests that some monosporous cultures of this strain 2 on slants contained a few sectors. Therefore in the selection of single spores from such cultures it would be difficult to obtain spores of the same genetic constitution. Such material would be of little or no value for studying variability.

H. sativum (1)

Single spore cultures of this strain of H. sativum were grown on different concentrations of the seven antibiotics under study. The number of variant colonies produced from these cultures are recorded in table XXXII. At each concentration a minimum of 125 colonies was examined. Compared with the checks the greatest number of colonies differeing in type from the parent are produced at the highest concentration of each antifungal antibiotic. This number in no instance, is very large. However, since practically

Table XXXII. The effect of antibiotics on the production of variant colonies from single-spore cultures of H. sativum (1) grown on different concentrations

Antibiotic	Concentration ppm.	Number of Variants
Gliotoxin		
	0	0
	1.0	2
	10.0	5
Griseofulvin		
	0	0
	1.0	0
	10.0	6
* Acti-dione		
	0	0
	1.0	2
	10.0	3
Candicidin		
	0	0
	0.1	1
	1.0	3

Table XXXII. continued

Antibiotic	Concentration ppm.	Number of variants
Nystatin		
	0	1
	0.1	1
	1.0	3
Tyrothricin		
	0	0
	25	2
	250	4
+ Streptomycin		
	0	0
	25	0
	250	0

* 250 colonies examined per concentration

\+ 200 colonies examined per concentration

no variants occur at 0 ppm. it would appear that the higher concentrations of the antifungal antibiotics used, namely gliotoxin, griseofulvin, acti-dione, candicidin, nystatin, and tyrothricin, induced the production of variants. Streptomycin, the antibacterial agent, at the three dilutions used produced no variants. This antibiotic had therefore little effect on variability. The general findings of this experiment were similar to those obtained in the sector test. Of the antibiotics tested griseofulvin appears to be particularly effective as a variant inducing agent and gliotoxin to approach it closely in this respect. Possibly if a larger number of colonies, eg. 500 to 600, had been examined a more positive indication might have been obtained of the relative effectiveness of the different antibiotics in variant production.

Mycelial characteristics were shown by most variant colonies. They were lighter in colour than their parent and produced abundant mycelium with a corresponding decrease in spore production. A few of the variants were white in colour but only one differed in zonation characteristics.

B. Colletotrichum linicolum (1) and (2)

Tests as in the previous investigations with single spore cultures were carried out using dilutions of the seven antibiotics.

No variant colonies appeared in plates inoculated from cultures of both strains grown on check media and on media containing antibiotic concentrations of gliotoxin, acti-dione, candicidin, nystatin, tyrothricin, and streptomycin.

A griseofulvin test was conducted similarly using a concentration range of 10, 1, and 0 ppm. In the test with the conidial strain 1 no variant types were found. However, in petri plates prepared from a single spore culture of strain 2, grown on 10 ppm. of this antibiotic, variants orange in colour and less mycelial in growth were observed. This was the only concentration of the three used at which variants occurred. The findings here corroborated the "sector" test results for strain 2 but not for strain 1. Yet, it was clear that under the action of griseofulvin strain 2 was more variable than strain 1.

C. Polyspora lini (1) and (2)

Experiments with strain 1 and strain 2 of this fungus were conducted as in previous tests just described for H. sativum and C. linicolum. A minimum of 150 colonies for each concentration was examined. The two strains will again be considered together as in the "sector" test.

The results for the production of variant colonies under antibiotic action are given in table XXXIII. An increase in variability with increase in concentration occurs in only two instances, in strain 1 under the action of griseofulvin and in strain 2 in contact with gliotoxin. By this method of measuring variability acti-dione, candicidin, nystatin, tyrothricin, and streptomycin were apparently unable to influence the variation of the two strains of P. lini tested. These results do not completely correspond with

Table XXXIII. The effect of antibiotics on the production of variant colonies from single-spore cultures of P. lini (1) and (2) grown on different concentrations

Antibiotic	Concentration ppm.	Number of Variants Strain 1	Strain 2
Gliotoxin			
	0	2	1
	0.1	2	1
	1.0	3	6
Griseofulvin			
	0	0	0
	1.0	0	0
	10.0	6	0
Acti-dione			
	0	0	1
	0.01	1	0
	0.1	0	0
Candicidin			
	0	0	2
	0.1	0	1
	1.0	0	2

Table XXXIII. continued

Antibiotic	Concentration ppm.	Number of Variants Strain 1	Strain 2
Nystatin			
	0	1	1
	0.5	0	0
	5.0	0	1
Tyrothricin			
	0	0	0
	25.0	0	0
	250.0	0	0
Streptomycin			
	0	1	0
	25.0	0	0
	250.0	0	0

those of the "sector" test, where candicidin and nystatin were shown to increase variation.

The variant colonies detected were similar in each test. Strain 1 variants differed in the distribution and amount of pigment, but no colonies of the strain 2 type appeared. Strain 2 variant colonies were lighter in colour than the original parent.

DISCUSSION

In the experiments described here six antifungal antibiotics gliotoxin, griseofulvin, acti-dione, candicidin, nystatin, tyrothricin and one antibiotic streptomycin, chiefly a bactericidal agent, have been used. Growth of the phytopathogenic fungi H. sativum, C. linicolum, and P. lini on artificial culture was inhibited by the antifungal antibiotics. Complete inhibition of growth was produced at different concentrations depending upon the antibiotic and the fungal species employed. Tyrothricin, for instance, was fungistatic to growth of the fungi at concentrations above 250 ppm. while in comparison no growth was detected after 5 days at dilutions above 10 ppm. of gliotoxin, candicidin, and nystatin. An extreme in antifungal activity was shown by acti-dione, which proved fungistatic to the growth of P. lini at 0.5 ppm. The antibacterial agent streptomycin had no appreciable effect on growth at the concentrations used. Therefore the antibiotics under study covered a wide range in potency.

Each antifungal antibiotic has been shown in one or more instances to increase the variability of the three phytopathogenic fungi whereas the antibacterial antibiotic streptomycin in no case affected variability at the concentrations used. It was observed through the use of dilution ranges that as the concentration was stepped up an increase in variability resulted. Where an effect on variation occurred, it was only at concentrations reducing fungal growth. Thus the factor influencing growth would appear to be similar or correlated to that affecting an increase in variation.

Some antibiotics influenced variability more effectively than others. The potency of an antibiotic to inhibit growth, however, gave no indication of its power to influence variation. For example, griseofulvin was the only antibiotic to affect the variability of all three fungi yet was less potent than gliotoxin, actidione, candicidin, and nystatin. In tests with H. sativum (1) and P. lini (1) this particular agent produced the greatest number of sectors recorded for these two fungi. It was the only antimicrobial agent to induce variability in both strains of C. linicolum. Brian, Curtis, and Hemming (7) have observed that griseofulvin produces abnormal development of fungal hyphae. This capacity of griseofulvin to affect variation may be connected with this characteristic. Under the action of gliotoxin, candicidin, and nystatin increased sectoring occurred in some strains of H. sativum and P. lini. Of these three antibiotics gliotoxin was the most effective in increasing variation, but its ability in this respect was less than

that of griseofulvin. Tyrothricin and acti-dione were noticed to affect only the variation of H. sativum (1).

From the results it would appear that the variability of different strains of a fungus differ in sensitivity to antibiotic influence. This was not surprising since under normal conditions (13), and also under the action of toxic substances (17, 47) Christensen and co-workers have shown that variation differs with the strain of H. sativum studied. In the experiments described here H. sativum (1) reacted in a similar manner with an increase in variability to all six antifungal antibiotics. Unfortunately, no measure of variation could be made on H. sativum (2). Strains of C. linicolum were affected differently by griseofulvin, strain 1 being less variable than strain 2 under the action of this antibiotic. The variability of P. lini strain 1 was increased in contact with griseofulvin, while strain 2 was more variable grown on artificial media containing gliotoxin.

Antibiotics influenced the production of different types of variants. All H. sativum variants differed in colour, zonation, spore and mycelial production, most of them being mycelial in form. C. linicolum (1) saltants were also mycelial in type. However, it would appear that a reversion in the normal type of saltation from conidial to mycelial forms occurred in C. linicolum (2) under antibiotic action, with the production of variants less mycelial in character than the parent. This trend of variation was corroborated by the variant colony test. P. lini on the other hand

produced variants which differed chiefly in colour, but retained the texture characteristic of the parent. In this species of fungus a definite pattern of saltation from strain 1 to strain 2 has been observed by Henry (24) and by the author on a few occasions. Nevertheless, antibiotics apparently did not affect this trend of saltation.

The results from the two methods employed to measure variation in most cases agreed in trend. In particular correspondence between results obtained for the variation of H. sativum (1) under antibiotic influence in the "sector" test and variant colony test was of interest. A few discrepancies were evident in the two sets of results from tests involving C. linicolum and P. lini. Under the action of griseofulvin a few mycelial sectors arose in C. linicolum (1), but no single-spore variant colonies of this type were obtained from single-spore cultures grown on similar antibiotic concentrations. Candicidin and nystatin were shown in some instances to increase variation, as indicated by the number of sectors produced, in some strains of P. lini. No such tendency was detected by the variant colony technique. Results from the variant colony experiments were in accordance with those of the sector test in all other experiments with these two fungi. The occurrence of these exceptions noted between results in these experiments may be explained by the fact that too few colonies were examined to show an effect on variation.

Part II

INTRODUCTION

Variation of imperfect fungi such as that reported in Part I may arise in several different ways.

1. Vegetative dissociation. This may occur due to assortment of nuclei when conidia are abstracted from conidiophores and when branching of mycelium takes place.

2. Vegetative association which is chiefly brought about through the process of anastomosis. Heterocaryosis or a state in which a binucleate or multinucleate cell carries genetically or structurally different nuclei, may thus arise.

3. Mutation, a process consisting of a spontaneous permanent change in the nucleus. The heterocaryotic condition can more rarely be initiated within a monocaryotic organism by a change of this type in one of the nuclei, which from that time on makes the mycelium heterocaryotic (22).

4. A parasexual cycle (32). In fungal hyphae diploid nuclei are sometimes formed and these at mitotic division give rise to recombinants.

Of the above possible mechanisms of variability only that of vegetative association through anastomosis was studied for the following reasons. 1) Previous work at other laboratories

with different fungi has drawn attention to its common occurrence (11, 18, 21, 25) and 2) an earlier work at this laboratory (12) indicated that antibiotic influence might stimulate this process in an easily observed form. Campbell (12) in 1934 detected a stimulation of the process of anastomosis between germinating spores of C. linicolum in the presence of a contaminant bacterium. It seemed possible that this stimulative action may have resulted from an antibiotic produced by the bacterium. If this could be demonstrated support would be provided for the hypothesis that anastomosis at least in part was responsible for the increase in variation shown in the studies in Part I.

The following studies were undertaken in an attempt to find a bacterium which would increase the frequency of anastomosis. The action of three antibiotics on the induction of this process was also investigated.

MATERIALS

Organisms studied

1. Five isolates of Colletotrichum linicolum designated A, B, C, D, and E were obtained from the sources listed in Table XXXIV.

Table XXXIV. Sources of Colletotrichum linicolum cultures

Culture	Source
A	A stock culture of this laboratory
B	The Science Service Botany and Plant Pathology Laboratory, Ottawa, Ontario, through the courtesy of M.E. Elliot, Assistant Mycologist
C	The Science Service Laboratory, Ste-Anne-de-la-Pocatiere, through the courtesy of Dr. R.O. Lachance
D	The Science Service Plant Pathology Laboratory, Winnipeg, Manitoba, through the courtesy of Dr. W.E. Sackston
E	Prof. A.E. Muskett, Plant Disease Division, Queens University, Belfast

2. Isolates of coliform bacteria Escherichia coli, Erwinia carotovora, E. amylovora, Aerobacter aerogenes, and A. cloacae were obtained from the sources indicated in table XXXV.

Table XXXV. Sources of the coliform bacteria used in the studies

Bacterium	Culture no. at source	Obtained from
Escherichia coli	PRL R 2	The National Research Council, Prairie Regional Laboratory, through the courtesy of Dr. A.C.Blackwood
" "	PRL R 25	
Erwinia carotovora	4493	Science Service Plant Pathology Laboratory, Winnipeg, Manitoba
E. amylovora	4008	
Aerobacter aerogenes	M - 148	National Research Council, Ottawa
" "	474	" " " "
Aerobacter cloacae	ATCC 529	" " " "
" "	ATCC 961	" " " "

3. Miscellaneous bacteria obtained from soil and flax seed.

GENERAL METHODS

The early studies on spore germination were made by means of a hanging drop technique using van Tieghem cells. However, a method employing slides having two concave wells each measuring 18 mm. in width and 1.75 mm. in depth was later adopted. For germination tests a measured drop of distilled sterile water or liquid medium was placed in each well, and the slide placed in a petri dish containing two layers of filter paper. Circular holes 2 cms. in width had been cut in the filter paper to correspond with the wells. This allowed microscopic examination of the drop culture in the well without removing the slide from the petri dish. The addition of a fixed quantity of water, 3 ml., to each dish ensured that the humidity was constant at the commencement of each experiment, and prevented the drying out of the drop culture. The bacteria and C. linicolum spores were then placed in the drop culture. To seal the petri dish petroleum jelly was applied to the rim of the inner plate. Germination tests were carried out in duplicate at room temperature, unless otherwise stated, and examined after 36 and 48 hours for evidence of anastomoses.

Bacterial and fungal cultures were maintained on potato dextrose agar throughout the studies. Ten-day old fungal cultures and 7-day old bacterial cultures were used.

EXPERIMENTS AND RESULTS

1) In these studies a method of obtaining bacterial isolates similar to that employed by Campbell (12) was used. It consisted of placing flax seeds in a spore suspension of C. linicolum (A), then planting them in soil. After 30 days the resulting seedlings were surface sterilized with mercuric chloride and placed on potato dextrose agar. Any bacterial colonies arising were then tested for their ability to stimulate anastomoses. Several isolates were obtained and tested but none gave the desired effect.

2) It appeared from Campbell's work that the particular isolate obtained could have come either from soil or from flax seed. Solutions containing a mixed microflora from these two materials, soil and flax seed, were prepared by placing samples of each in respective test tubes containing sterile distilled water. Drops from these two solutions were placed in the wells and inoculated with C. linicolum (A) and incubated for 36 to 38 hours. No anastomosing was observed to take place.

3) As no effect was observed in the last test where a mixed population of microorganisms from soil and flax seed was used, the isolation of a large number of individual bacteria from these two possible sources was undertaken. For this purpose several media were employed: potato dextrose agar pH 7, and 5, and nutrient agar at pH 6.8. By a dilution technique 322 isolates were obtained from 5 samples of Edmonton soil and a large number of flax seed samples.

Each of these isolates was tested in turn for its ability to stimulate anastomosis. Only on a very few occasions was this process observed to occur; in each case a single fusion bridge being observed. On repeating these particular experiments no confirmation was obtained that this was the result of bacterial action.

Other effects were observed. Antagonism was manifest in certain cases by complete inhibition of germination and in others in the reduction of germination. Complete inhibition was affected by 8 of the bacterial isolates. Abnormal germination evidenced by the formation of "vesicle shaped" structures on the germ tubes and resulting hyphae was produced by 7 of the bacteria. A similar effect has been observed by Lachance (26) in previous studies on C. linicolum and by Ward (40) in H. sativum.

4) Through communication with the original author it was learned that the bacterium isolated had been tentatively classified as belonging to the coliform group. Coliform bacteria, Escherichia coli, Erwinia carotovora, Erwinia amylovora, Aerobacter aerogenes, and Aerobacter cloacae were therefore obtained and tested but failed to produce an increase in anastomoses.

5) At this stage of the investigation, since no positive results had been obtained, it was thought possible that under certain specific modified conditions a particular bacterial isolate might induce anastomosis between fungal spores. A study of the effect of temperature, pH, and media on anastomosing was therefore made.

a) The effect of temperature

A preliminary test was carried out on the effect of temperature on the germination of C. linicolum (a). Water drops containing spores of the fungus were placed in incubation cabinets maintained at constant temperatures over a range of 5° C. to 35° C. At least 300 spores were examined per drop culture.

Table XXXVI. The effect of temperature on the germination of C. linicolum (A)

	Temperature °C.						
	5	10	15	20	25	30	35
Percentage of germination	6	74	85	89	95	42	0

Percentage given is an average of two cultures.

From the results in table XXXVI it is evident that germination occurs over a wide range of temperature. The optimum temperature for spore germination is evidently at or near 25° C. Above this temperature germination is extremely reduced with little rise in temperature, whereas in comparison with decrease in temperature a gradual reduction in germination takes place. The results obtained for germination compared favourably with those obtained by Campbell (12).

Each of thirty chosen bacterial isolates was placed in water drop cultures containing fungal spores and kept for 36 to

48 hours at the different temperatures indicated in table XXXVI.

The bacterial isolates under temperature conditions which influence germination greatly were unable to increase anastomosing. An occasional fusion was observed in certain cultures but on further testing this did not appear to be due to the temperature effect nor to the action of the bacterium.

b) The effect of pH

Solutions having a pH reaction of 4, 6, 7, 8, and 10 were prepared using N/10 NaOH and N/10 HCl. The bacterial isolates together with C. linicolum spores were placed in water drops having the above pH reactions. No estimation of germination percentage was taken. Under these conditions no increase in anastomosing was observed.

c) The effect of media

It was reported by Bourchier (5) that a particular fungal isolate, a member of the Thelephoraceae, produced more hyphal fusions on potato dextrose agar than on carrot dextrose agar. It also grew more poorly on the former than on the latter type of medium. C. linicolum in contrast grew well on potato dextrose agar (table XXXVII). It was therefore thought that anastomosing might be encouraged by maintaining it on a medium less optimum for growth than potato dextrose agar.

C. linicolum (A) was transferred and maintained on three different media, potato dextrose agar, carrot dextrose agar, and

nutrient agar. On the latter two media reduced growth occurred compared with that on carrot dextrose agar (table XXXVII). After three repeated transfers on these media fungal spores were placed in water drop cultures together with the bacterial isolates chosen and observed for an increase in anastomoses, but none was noted.

No difference in the number of fusion bridges produced was observed when fungal spores and bacteria were placed in either distilled water or in nutrient solution drop cultures.

In one series of experiments the difference between solid and liquid media on the inducement of anastomisis was investigated. Lachance (26) reported a difference in antagonistic effect in certain instances when solid and liquid media were used. The solid media, consisting of water agar plugs cut from poured plates, was placed on a slide contained in a petri dish set up as in other tests. No anastomosing was observed under either set of conditions.

6) Since no stimulation of anastomosis had been observed in previous experiments, it was thought that a strain difference might exist, the strain A available at this laboratory having no potential tendency for its germinating spores to fuse. Consequently four other C. linicolum isolates were obtained from different sources for comparison. The five isolates designated A, B, C, D, and E were grown on potato dextrose agar, carrot dextrose agar, and nutrient agar and differences in growth rate were established as shown in table XXXVII.

Table XXXVII. The growth of the five isolates of C. linicolum on different media as measured by the colony diameter in mm.

Isolate	Media		
	PDA	CDA	NA
A	41	38	27
B	20	19	18
C	34	31	21
D	33	28	24
E	32	30	28

PDA - potato dextrose agar, CDA - carrot dextrose agar, NA - nutrient agar.

It was also observed from colony characteristics on the three media that the isolates differed from each other. B, C, and D were conidial in type whereas A and E were mycelial in character. A and B were at the two extreme ends of the scale in colony characteristics.

The above isolates A, B, C, D, and E were individually placed in contact with each of the thirty chosen bacterial isolates obtained from soil and flax seed. There was no apparent effect on anastomosing. The action of these bacteria on a mixed inoculum consisting of spores from each fungal isolate was studied

in the hope that some evidence of anastomosis between spores of the different fungal isolates might be gained. Similar information was sought by placing A, B, C, D, E in combinations of pairs with each of the bacteria. No evidence of increased anastomosing was obtained in any of these experiments conducted.

7) Antibiotics griseofulvin, acti-dione and streptomycin were made up in solution, as described in part I of this investigation, at the concentrations indicated below.

1. Griseofulvin 50, 20, 10, 1, and 0 ppm.
2. Acti-dione 10, 5, 1, 0.1 and 0 ppm.
3. Streptomycin 250, 100, 10, 1 and 0 ppm.

Griseofulvin was chosen since in variation studies it proved to be the most active agent. Antibiotics acti-dione and streptomycin were included because of their widespread use.

Only strains A and B of C. linicolum were employed in these experiments.

Reduction in germination with increase in concentration of griseofulvin and acti-dione was observed but streptomycin had no such effect. Griseofulvin was found to produce the abnormal hyphal development and germination known to occur under the action of this antibiotic. No increase in the number of fusions was detected.

DISCUSSION

Attempts to repeat Campbell's results, namely to induce anastomosis between spores of C. linicolum, were unsuccessful. Conidial anastomosis has been reported in other species (13, 44). However, reports of fusions between fungal spores are few compared with those involving hyphae. It is possible that hyphal fusions may differ in nature to those formed between germinating spores. Thus, although no increase in anastomosis was demonstrated by the action of bacteria or antibiotics in the technique employed it might be induced between hyphae. It is recognized by the author that the bacterium isolated by Campbell (12) probably was not encountered in these studies.

GENERAL DISCUSSION

Previous work having shown that a number of toxic substances will increase variability in fungi (15, 47) it seemed possible that antifungal antibiotics might have a like effect. This has been amply confirmed in these studies. The antibiotics under investigation were produced by a wide variety of organisms and differed appreciably in their toxic effect on fungal growth. Only antibiotics having a toxic effect, namely the antifungal agents, and at concentrations toxic to growth influenced variation. Although increase in variability appeared to be associated with growth inhibition another factor of fungal strain difference was apparently involved. Thus the extent to which variation was influenced depended upon the antibiotic, the concentration of the antibiotic, and the strain of fungus used.

No pathogenic studies were carried out on the variants obtained in these studies. Other workers have, however, obtained variants differing in pathogenicity as a result of culturing pathogenic fungi on media of various kinds, including some containing toxic substances. Christensen (13) observed in early pathogenic studies of H. sativum grown in culture that the variants obtained did not all possess the same degree of virulence. Most of them were similar to the parent but two were decidely more virulent than the respective parents. Christensen concluded that change in virulence of this type was probably occurring continually in nature. Also in investigations with a toxic substance produced

by Bacillus mesentericus Christensen and Davies (17) reported induced H. sativum variants having a greater degree of virulence than the parent. In the case of C. linicolum Schwinghamer (33) has noted that sparse sporulation is associated with loss in pathogenicity. In the present studies C. linicolum strain 2 produced saltants which sporulated more abundantly than their parents. If pathogenicity is correlated with degree of sporulation such variants would be expected to be more pathogenic than the original form of C. linicolum strain 2, though this has not been demonstrated. Also, in the case of the third fungus studied, namely P. lini, it was found by Henry (24) that some of the normal saltants produced from the black soft form of this fungus were more pathogenic than their respective parents. It appears quite probable that variants produced under antibiotic action may differ in pathogenicity but this remains to be demonstrated.

The fact that antibiotics increased variability in culture is no proof that these compounds will increase variation in nature. Evidence is now available that microorganisms produce antibiotics in their natural habitats. Gliotoxin (46) and griseofulvin (45) have been shown to be produced in soil, while patulin (9) has been isolated from diseased apple tissue. Attempts to detect some antibiotics in soil have been unsuccessful. Stevenson (35) has suggested that this failure may be due to the methods used and that the agents may still be present in sufficient concentration in localized areas to exert an influence. The ecological significance is evident when consideration is given to the

role antibiotics might play in altering and deciding the microflora composition through an increase in the number of variants. Thus variation in nature, because of these substances, may be greater than is realized.

In practical control antibiotics would be applied at concentrations which will destroy or inactivate pathogenic microorganisms. Under certain conditions, however, dilution of these concentrations might well occur to an extent permitting limited growth to take place. At such concentrations an effect on variability might come into play. Evidence along this line is provided by the work of Aytoun (2) on the effect of griseofulvin on phytopathogenic fungi in vivo. Plants sprayed with 85 ppm. of griseofulvin were assayed and found to contain in certain parts a concentration of 2-3 ppm./gm. live weight. Such a concentration approaches that shown to affect variation in the experiments described here. Thus an antibiotic applied at a concentration sufficiently high to control a disease organism externally, might in the plant, because of dilution, increase its variability.

Also, the fact that microorganisms differ in their sensitivity to antibiotics should be taken into consideration. An antibiotic that may successfully control one species may only reduce the growth of another, or have no effect at all. In this way an antibiotic applied to control one organism may increase the variability of others.

The studies on anastomosis would tend to indicate that this process is not an important mechanism in increasing variation, which was found in the studies to arise from antibiotic action. If it does play a part it would appear to be a very limited one. Lachance in studies of the antagonism of soil microorganisms on C. linicolum spores tested in the region of four hundred microbial isolates, yet no anastomosis was reported. If anastomosing is unimportant other mechanisms, or another mechanism, must be responsible for the increased variability observed. Certain chemical substances have been shown to increase mutation, and one substance has been shown to stimulate the formation of diploid nuclei, which give rise to recombinants through the parasexual cycle (32). Antibiotics may affect these two processes. Christensen and Davies (17) suggest that variability induced in H. sativum by a toxic substance produced by Bacillus mesentericus is the result of mutation followed by selection. The toxic substance provides conditions which enable the mutant to survive and successfully compete with the parent. In a cytological study Christensen and Davies (16) concluded that the occurrence of variants in H. sativum was mainly attributable to new mutations since only rarely did more than one nucleus enter the young conidiophore and conidium. However, from other cytological studies of this fungus Hrushovetz (25) states that a mechanism exists for the perpetuation of heterokaryons. Hyphal fusions occur frequently and several nuclei invariably enter the young conidium. Little or no cytological work has been carried out on either C. linicolum or P. lini.

SUMMARY

1. Six purified antifungal antibiotics gliotoxin, griseofulvin, acti-dione, candicidin, nystatin, tyrothricin and one antibiotic streptomycin, widely used as an antibacterial agent, were tested in vitro for ability to influence the variation of strains of three phytopathogenic fungi, namely, Helminthosporium sativum, Colletotrichum linicolum, and Polyspora lini. The variability was measured by the number of sectors produced, and by the number of variant colonies which arose from single spore cultures.

2. All antifungal antibiotics were observed in one or more instances to increase variability in the three fungi. With increase in concentration an increase in variability resulted. Increase in variation occurred only when reduced growth took place. Low concentrations of antibiotics had no tendency to increase variation.

3. Increase in variation frequently differed with the antibiotic, the concentration of antibiotic, and the strain of the fungus used.

4. Streptomycin, an antibiotic mainly antibacterial in action, had no apparent effect on variability.

5. The antifungal antibiotics inhibited the growth of all three fungi on potato dextrose agar. Complete inhibition of growth occurred at different concentrations depending on the antibiotic. A relative reduction in growth took place with increase in concentration. Streptomycin had little effect on growth at the concentrations used.

6. The order of increasing antibiotic sensitivity was H. sativum, C. linicolum and P. lini.

7. Results from both the "sector" test and "variant colony" test corresponded in trend, with a few exceptions.

8. The normal trend of variation from conidial to mycelial type was observed in H. sativum (1) and C. linicolum (1). However, in C. linicolum (2) a mycelial form, there was an apparent reversion in pattern with production of variants less mycelial in character. Antibiotics were unable to induce the normal sporulating form of P. lini (strain 1) to give rise to the variant mycelial form (strain 2).

9. Some antibiotics at higher concentrations stimulated mycelial and pigment production in strains of C. linicolum.

10. Many individual bacterial isolates from soil and flax seed, together with five bacteria from the coliform group, failed to stimulate the process of anastomosis between spores of C. linicolum. Similar results were obtained when some of the isolates were tested under varied conditions of temperature, pH, moisture, and media. No differences were found between strains or mixtures of strains of C. linicolum in the production of fusion bridges when placed in the presence of bacterial isolates. Complete and partial inhibition of germination, also abnormal germination results from the treatments. The antibiotics griseofulvin, actidione, and streptomycin also failed to stimulate anastomosis in C. linicolum.

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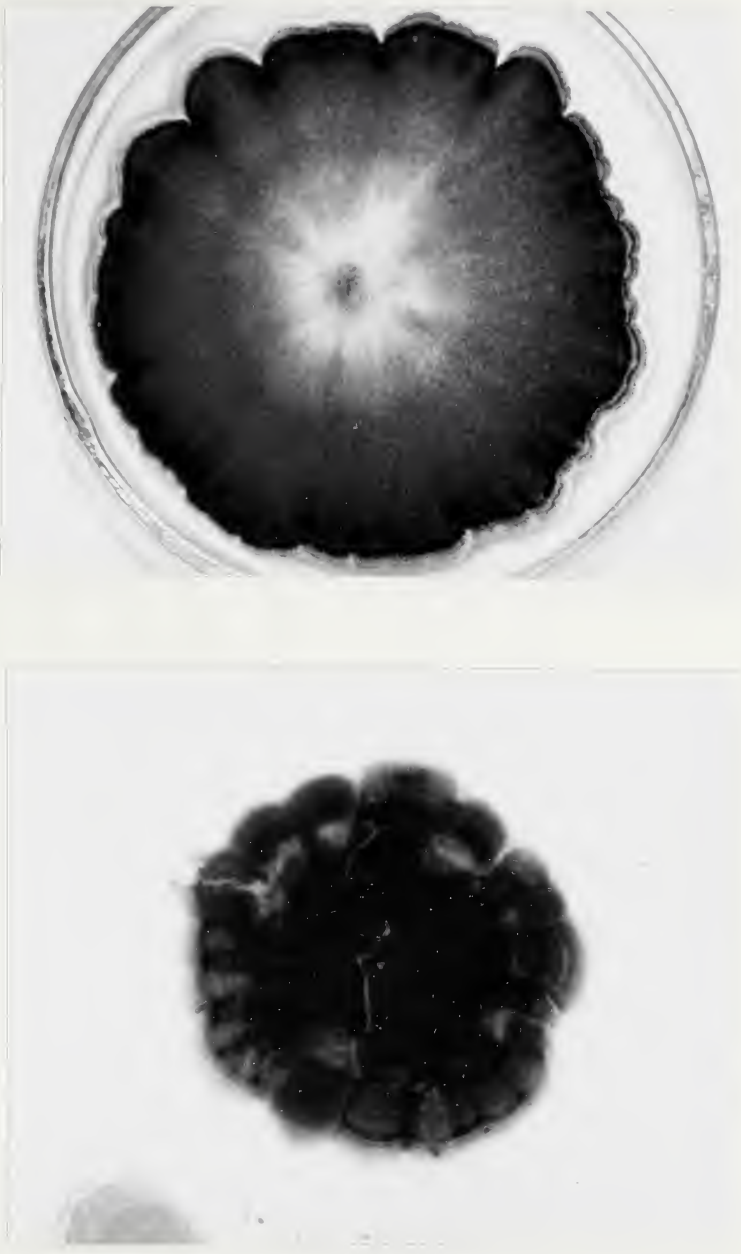

Figure 1 The sectoring of H. sativum (1) when grown on potato dextrose agar containing dilutions of gliotoxin. Upper plate 0 ppm. Lower plate 10 ppm.

Figure 2 Shows the extreme variability of H. sativum (2), in the production of a large number of sectors, when grown on a check medium (potato dextrose agar).

Figure 3 Shows a mycelial sector in a colony of C. linicolum (1) grown on potato dextrose agar containing 5 ppm. of griseofulvin.

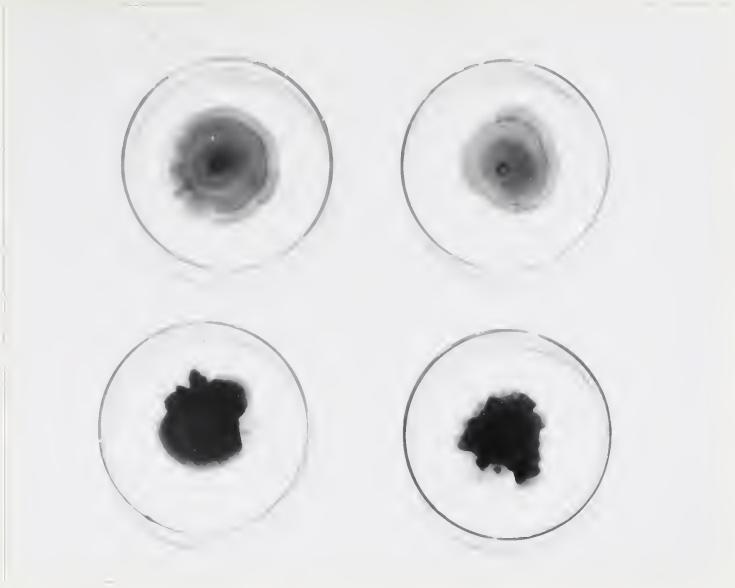

Figure 4 The sectoring of P. lini (2) when grown on potato dextrose agar containing dilutions of gliotoxin. Upper row from left to right 0 and 0.1 ppm. Lower row from left to right 0.5 and 1.0 ppm.

Figure 5 Colony of P. lini (1), the black soft form, showing a sector of P. lini (2), the grey tough form.